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INVITED REVIEW: FOREST BIOTECHNOLOGY: INNOVATIVE METHODS, EMERGING OPPORTUNITIES

NARENDER S. NEHRA, MICHAEL R. BECWAR, WILLIAM H. ROTTMANN, LESLIE PEARSON, KAMAL CHOWDHURY, SHUJUN CHANG, H. DAYTON WILDE, ROBERT J. KODRZYCKI, CHUNSHENG ZHANG, KATRINA C. GAUSE, DAWN W. PARKS, AND MAUD A. HINCHEE*

ArborGen, P.O. Box 840001, Summerville, SC 29484-8401

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Summary

The productivity of plantation forests is essential to meet the future world demand for wood and wood products in a sustainable fashion and in a manner that preserves natural stands and biodiversity. Plantation forestry has enormously benefited from development and implementation of improved silvicultural and forest management practices during the past century. A second wave of improvements has been brought about by the introduction of new germplasm developed through genetics and breeding efforts for both hardwood and conifer tree species. Coupled with the genetic gains achieved through tree breeding, the emergence of new biotechnological approaches that span the fields of plant developmental biology, genetic transformation, and discovery of genes associated with complex multigenic traits have added a new dimension to forest tree improvement programs. Significant progress has been made during the past five years in the area of plant regeneration via organogenesis and somatic embryogenesis (SE) for economically important tree species. These advances have not only helped the development of efficient gene transfer techniques, but also have opened up avenues for deployment of new high-performance clonally replicated planting stocks in forest plantations. One of the greatest challenges today is the ability to extend this technology to the most elite germplasm, such that it becomes an economically feasible means for large-scale production and delivery of improved planting stock. Another challenge will be the ability of the forestry research community to capitalize rapidly on current and future genomics-based elucidation of the underlying mechanisms for important but complex phenotypes. Advancements in gene cloning and genomics technology in forest trees have enabled the discovery and introduction of value-added traits for wood quality and resistance to biotic and abiotic stresses into improved genotypes. With these technical advancements, it will be necessary for reliable regulatory infrastructures and processes to be in place worldwide for testing and release of trees improved through biotechnology. Commercialization of planting stocks, as new varieties generated through clonal propagation and advanced breeding programs or as transgenic trees with high-value traits, is expected in the near future, and these trees will enhance the quality and productivity of our plantation forests.

Key words: conifers; hardwoods; somatic embryogenesis; transformation; gene discovery; plantation forestry; tree genomics.

Introduction

Forest trees are important for our environment, as a source of timber and a range of other products in our daily lives. Conventional silvicultural practices and breeding techniques have contributed significantly to the improvement of forest tree species in the past, and will continue to have a substantial impact on the genetic gain and productivity of economically important tree species by providing better germplasm and improved management practices for plantation forests. Traditional breeding methods are often constrained by the long reproductive cycles of most tree species and the difficulty in achieving significant improvements to complex traits such as wood properties, disease and pest control, and tolerance to abiotic stresses. Biotechnology is an adjunct to the long-established traditional tree improvement practices and one that utilizes fundamental discoveries in the field of plant tissue culture for clonal forestry, gene transfer techniques, molecular biology, and genomics. These new discoveries now provide an extended platform for improvement of traits that have previously been considered impractical via conventional breeding methods. Biotechnology provides exciting opportunities to further expand our understanding of genome organization and functioning of genes associated with complex value-added traits, and to transfer such genes into economically important tree species. This will lead to the development and deployment of trees ready to meet the future demand of the world's ever-increasing population for timber and other forest products, while preserving natural forests for future generations.

Several recent review articles have amply covered the role of biotechnology in plantation forests (Fenning and Gershanzon, 2002), economic benefits resulting from the introduction of forest biotechnology (Sedjo, 2001), and ecological issues associated

*Author to whom correspondence should be addressed: Email mahinch@arborgen.com

with the deployment of genetically modified forest tree species (van Frankenhuyzen and Beardmore, 2004). In this review article, we cover recent innovative technologies, which will provide the basis for acceleration in the improvement of forestry through biotechnology. Topics that are specifically addressed in this review include the recent exciting developments in the field of conifer somatic embryogenesis (SE) for large-scale cloning and deployment of elite germplasm, in transformation methods for insertion and expression of value-added traits into elite clones, and in molecular biology and genomics efforts for improvement of forest tree species.

Conifer Plant Regeneration and Transformation

Trees that will supply the vast majority of the future world need for wood, fuel, paper, and other wood-derived products will come from highly productive, managed tree plantations rather than natural stands. Conifers, primarily *Pinus* spp., *Picea* spp., and *Pseudotsuga* spp. are the most widely planted timber and pulpwood species, covering over 60% of the plantation forests worldwide, while hardwoods, primarily *Eucalyptus* spp. and *Populus* spp., account for the remainder of plantation forests (FAO, 2000). *Pinus* (pine), *Picea* (spruce), and *Pseudotsuga* (Douglas-fir) plantations are common in Canada and the Pacific Northwest of the US. Among the conifer species planted in the US, loblolly pine (*Pinus taeda*) is by far the most important in terms of number of seedlings planted. Radiata pine (*Pinus radiata*) is extensively planted in Chile, New Zealand, and Australia. Long-rotation conifers such as Norway spruce (*Picea abies*) and Scots pine (*Pinus sylvestris*) are species widely planted in the temperate regions of Asia, Europe, and North America. All of these economically important conifer species have been the target for plant regeneration and genetic modification research.

Conifer Somatic Embryogenesis

Pine plantations have been primarily established using seeds obtained from wild trees or harvested from open-pollinated seed orchards. Although some seed is now obtained from controlled pollination between selected parents, there is still a wide variability in the performance of each individual tree within a plantation. The planting of superior trees with respect to yield, form, and tolerance to pathogens will be necessary in order to achieve the desired productivity gains. The asexual propagation of selected elite conifer genotypes has been a significant research effort in the forestry community to assist in the deployment of improved genetics in large-scale plantations. Propagation technologies have focused on micropropagation of shoot cultures, rooted micro- or macrocuttings and on SE. Hardwood trees have proven to be more amenable to micropropagation and rooted cutting techniques. However, propagation by rooted cuttings is not cost effective on a large scale with most conifers. SE is a technology that promises to deliver high-volume, cost-effective production of genetically selected elite varieties.

SE of conifers has been researched, developed, and significantly improved in the last 20 years. Recent reviews, including those by Park et al. (1998), Cyr and Klimaszewska (2002), Jones (2002), Klimaszewska and Cyr (2002), Park (2002), and Sutton (2002), concentrated on scale-up of conifer SE and its potential application to clonal forestry. Reviews by Stasolla et al. (2002), von Arnold et al. (2002), and Stasolla and Yeung (2003) covered improvements in development and maturation of conifer somatic embryos, including embryo quality. Since these reviews, significant advancements have been made toward the commercial application of conifer SE.

Conifer SE is a multistep process and the success of each step depends on the success of the preceding steps. The main steps are: initiation, embryogenic tissue proliferation, somatic embryo maturation (morphological and physiological), embryo harvest and storage, and embryo conversion into planting stocks. A key advantage of conifer SE over other micro- and macropropagation methods is that the embryogenic cultures can be cryopreserved and stored while field tests are conducted to identify genotypes with significant genetic gain (Park et al. 1998). This advantage alleviates the negative age-dependent effects on propagation associated with vegetative cuttings of some conifer species. Embryogenic cultures also provide the advantage of being amenable to current transformation methods for adding value-added genes. Here, we review the advances made in conifer SE with a focus on information relevant to commercial scale up. In addition, a survey of patents in conifer SE is presented, as many of the technical advances are described in patent applications rather than in scientific publications.

Initiation of SE in conifers. Critical factors in initiation of SE in conifers are the explant type and the developmental stage of the particular explant tissue. Additionally, genotype plays an important role, showing that SE is under strong genetic control (Park, 2002). Explants from conifer seeds (i.e. zygotic embryos) have been the most responsive for SE. The responsive embryo developmental stage for both immature and mature embryo explants has been much broader among *Picea* species compared with *Pinus* species.

Much of the work on improving initiation of SE in *Pinus* species, many of which are of significant economic importance and also have relatively low initiation frequencies, has focused on modification and optimizing culture medium using immature zygotic embryos or whole immature megagametophytes (with intact developing zygotic embryos) as starting explants (Arya et al., 2000; Mathur et al., 2000; Percy et al., 2000; Miguel et al., 2004). Lower auxin levels [4.5 μM rather than 13.6 μM 2,4 dichlorophenoxyacetic acid (2,4-D)] increased initiation frequency in *Pinus strobus* (Klimaszewska et al., 2001a). Use of brassinolide (Pullman et al., 2003) or the gibberellin inhibitor, paclobutrazol (Pullman et al., 2005), has also been shown to improve initiation in *Pinus taeda.* There has been limited recent work on initiation from mature zygotic embryo explants of *Pinus* species (Radojevic et al., 1999; Tang, 2001). Even though mature seed explants provide an advantage of being able to initiate embryogenic cultures at any time of the year, a field test is still required to identify superior genotypes, just as with immature seed explants, before large-scale production can be employed with select, superior clones (Park et al., 1998).

Conifer species are generally considered to be more recalcitrant than angiosperms for SE from vegetative tissue explants obtained from mature trees. However, some progress has been made recently on cloning via SE from mature conifer tree tissues. Bonga (1997) first reported the importance of a cold treatment of mature conifer explant tissue for the initiation of embryo-like structures in *Larix*. Further improvements in *Larix* SE from mature explants have been reported (Bonga, 2004). A cold treatment of vegetative bud tissue from mature trees was also important to successful SE initiation in *Pinus patula* (Malabadi and van Staden, 2003, 2005) and *Pinus*

kesiya (Malabadi et al., 2004). A cold treatment of apical dome sections or cultures was also reported to induce further development of early-stage pine somatic embryos (Deb and Tandon, 2004a, b).

Compared with pines, much more progress has been made with spruce on initiating SE from mature trees. The genetic fidelity of plants regenerated via SE from explants of mature trees is often questioned. Harvengt et al. (2001) examined genetic stability of Norway spruce trees regenerated over an extended time (up to 3 yr) from mature trees. Using micosatellite DNA markers, they found no allelic difference at six loci between 3-yr-old Norway spruce trees regenerated through SE from needles compared with the 6-yr-old control (source) tree. This molecular evidence, although limited, is consistent with true-to-type cloning; furthermore, it was in agreement with a lack of phenological differences between the source tree and the 3-yr-old secondary tree derived from it. Bud break and flushing, shoot height, and diameter were all in the same range as the controls. Helmersson et al. (2004) also found high stability of microsatellite loci during early stages of SE in Norway spruce.

Production of high-quality somatic embryos. Although improvements have been made in the initiation and proliferation of conifer embryogenic tissue, the morphological and physiological maturation attained by somatic embryos are often not optimal for efficient germination, and research towards improving these steps is needed. By definition, a quality somatic embryo refers to a somatic embryo that readily converts into a normal plant. It is generally assumed that an embryo may acquire its quality during the maturation step. Recent studies suggest that pro-embryos at the proliferation step and acquisition of desiccation tolerance play an important role in embryo quality. Filonova et al. (2000a) demonstrated that each pro-embryo mass (PEM) passes through a series of three characteristic stages (I, II, III) to transdifferentiate into a somatic embryo. Embryo development is triggered by arresting tissue proliferation due to removal of plant growth regulators (PGRs) such as 2,4-D and benzyladenine (BA) from the medium and continues with the availability of abscisic acid (ABA) in the maturation medium. The removal of the PGRs is a key developmental switch for PEM-to-somatic embryo transition and careful manipulation of PGRs prior to ABA application may be critical for embryo production and enhancement of embryo quality (Bozhkov et al., 2002). It is, therefore, critical to maintain an environment in the proliferation medium that allows the growth of maximum numbers of normal stage III PEMs. Filonova et al. (2000b) also demonstrated that two waves of programmed cell death (PCD) occur during formation and development of somatic embryos in Norway spruce. The first wave of PCD is responsible for the degradation of PEMs when they give rise to somatic embryos and the second wave of PCD eliminates terminally differentiated embryo-suspensor cells during early embryogeny.

During the maturation phase, optimization of ABA levels, control of ethylene biosynthesis, imposition of osmoticum, and drying treatments have been shown to influence somatic embryo quality (Stasolla and Yeung, 2003). Excess ethylene in the maturation medium environment can negatively affect the number and quality of somatic embryos, and application of ethylene inhibitors in the medium had opposite effects in white spruce (El Meskaoui et al., 2000). Partial and slow desiccation improved the germination of spruce somatic embryos (Roberts et al., 1991). Acquisition of desiccation tolerance is achieved by imposing osmotic stress on white spruce cultures using increased levels of polyethylene glycol (PEG) and sucrose (Attree et al., 1991; Stasolla and Yeung, 2003). Recently, Stasolla et al. (2004) demonstrated that inclusions of reduced glutathione in the maturation medium increased the conversion frequency of white spruce somatic embryos without the need of a partial drying treatment. This beneficial effect was the result of major alterations in morphology and gene expression during the maturation period. Similarly, a desiccation step was not needed when *P. strobus* somatic embryos were matured on medium containing a high percentage (1%) of Gelrite (Klimaszewska et al., 2000).

Recently, the relationship of changes in carbohydrate status and cold treatments to desiccation tolerance of spruce somatic embryos has been examined (Bomal et al., 2002; Lipavska et al., 2003). The role of carbohydrate metabolism in conifer SE is reviewed by Lipavska and Konradova (2004). They propose several hypotheses, including that raffinose oligosaccharides are substantially responsible for desiccation tolerance acquisition and/or improved germination in spruce somatic embryos. Sucrose, in addition to being an important energy source and osmotic agent, may serve a developmental signaling or regulatory role in conifer SE development (Iraqi and Tremblay, 2001a, b).

Pond et al. (2002) improved tolerance of *Picea glauca* somatic embryos to rapid desiccation after acclimation with a cold treatment. The optimal response was obtained with somatic embryos grown for 51 d (cotyledonary stage) on maturation medium and subsequently exposed to 5°C for 8 wk prior to 2 h air-drying. Desiccation tolerant *P. abies* somatic embryos were also produced in liquid culture medium containing PEG and ABA (Gorbatenko and Hakman, 2001). These somatic embryos tolerated desiccation (reduction in relative humidity to 31%) and regenerated plantlets with improved morphology compared with non-desiccated controls.

Most of the above reports deal with *Picea* species, while much less fundamental work on desiccation tolerance has been done with *Pinus* species. Desiccation tolerance was induced by maturing somatic embryos of loblolly pine (*P. taeda*) on medium with ABA and/or PEG (Tang, 2000). Interestingly, desiccation-tolerant somatic embryos had higher peroxidase activity compared with non-desiccation-tolerant somatic embryos. The author hypothesized that higher peroxidase activity of desiccation-tolerant somatic embryos may have allowed them to catalyze the reduction of H_2O_2 produced by drought stress, and protected them from oxidative damage.

Conifer somatic embryos lack the presence of the nutrient-rich megagametophyte that surrounds the zygotic embryo during germination and seedling establishment. Research has been conducted to determine the requirements for the provision of additional nutrients during somatic embryo germination. Klimaszewska et al. (2004) characterized differences in moisture content and storage protein accumulation in somatic and zygotic embryos of *P. strobus*. Moisture content of somatic embryos was slightly higher and storage proteins were 30–50% lower compared with mature zygotic embryos. A better understanding of events controlling somatic embryo quality will most likely improve germination and conversion and also aid in the implementation of synthetic or 'manufactured' seed as a deployment option.

Improving quality of planting stock. Establishment of seedlings in nursery beds is the forest industry accepted standard for seedling production of pines in the southeastern US. Approximately one billion seedlings are produced for reforestation per year (McKeand et al., 2003), and most of these are established and grown to

planting stock in nursery beds. Conifer seeds are an ideal propagule because they tolerate desiccation, can be stored in the dried state, and germinate and grow to seedlings at high frequencies. Progress has been made to emulate and characterize the mechanisms that enable conifer somatic embryos to have these same desirable traits.

Hogberg et al. (2001) examined *in vitro* culture factors that influenced subsequent growth of somatic embryo plants of Norway spruce (*P. abies*). Increased duration of exposure to ABA and light resulted in shorter somatic embryo-derived plants. In a related study, Hogberg et al. (2003) used certain selection criteria at *ex vitro* transfer to identify somatic embryo plants with height growth characteristics comparable with those of seedlings. Epicotyl length and presence of lateral roots proved to be important parameters for selection. The growth of somatic embryo plants selected in this way was similar to that of seedlings. Thus, *in vitro* optimization and selection showed promise to improve performance of SE clonal planting stock.

Scale-up of conifer SE planting stock production via direct sowing of manufactured seed (often referred to as artificial or synthetic seed) in nursery beds is one deployment option that is being researched and developed at a commercial scale. This scenario interfaces with existing nursery production practices for conifer bare root seedlings. A recent article on biotechnology in forestry (Weyerhaeuser, 2003) outlines a vision for combining cloning via SE, manufactured seed, and automation to achieve cost-effective planting stock production.

An increase in patent publications on conifer SE technology reflects both the maturing of the technology and its potential for large-scale commercial production. A survey of US patents issued or published applications (www.uspto.gov) on conifer SE revealed a total of 80 patents and published applications since 1990. Figure 1 shows a distribution of these patents by the primary subject matter or step of the SE process. The highest number of patents and applications (a total of 23) are on embryo production methods and manufactured seed (a total of 17). About 70% (13 of the 19) of recent applications have been devoted to improving embryo production and ways to characterize or improve embryo quality. This increased activity in solving embryo production and quality issues reflects an understanding that making significant improvements in these areas will not only increase efficiency but also decrease planting stock production costs.

Conifer Transformation

Successful application of biotechnological tools for improvement of conifer species requires efficient gene transfer systems. The development of reliable and reproducible regeneration systems via SE for the economically important conifers has paved the way for the advancement of gene transfer technology and also for the development of cost-efficient avenues for deployment of genetically improved planting stocks. Unlike most agronomically important angiosperms, gymnosperms were considered to be outside the natural host range of *Agrobacterium tumefaciens* for gene transfer. Therefore, the initial efforts for gene transfer in conifers were focused on the use of DNA-coated microprojectile bombardment (biolistics) methods for recovery of transgenic plants (Table 1). The first stable transformation in a commercially important conifer species (*P. glauca*) was achieved via particle bombardment of embryogenic callus tissue (Ellis et al., 1993). This protocol was successfully adapted for transformation of other species of spruce (Charest et al., 1996; Walter et al., 1999), larch (Klimaszewska et al., 1997) as well as radiata pine (Walter et al., 1998) for regeneration of stably transformed embryogenic tissue and plants. The application of biolistics to loblolly pine required the identification of the appropriate stage of somatic embryos, the development of suitable culture media for maintenance, the enrichment of target tissue prior to bombardment, and the successful recovery of cells capable of producing transgenic tissue and plants post bombardment (Kodrzycki et al., 2002). Further optimization of biolistics parameters and selection strategies has allowed the regeneration of transgenic plants from elite loblolly germplasm of diverse genetic backgrounds (Connett et al., 2002).

Recently, *Agrobacterium*-mediated transformation has become the method of choice for gene transfer in conifers (Table 1), primarily owing to the ease of transformation, high efficiency, and cleaner integration of T-DNA into the host genome. Several factors such as efficient regeneration systems, design of enhanced gene expression vectors, optimization of parameters for *Agrobacterium*

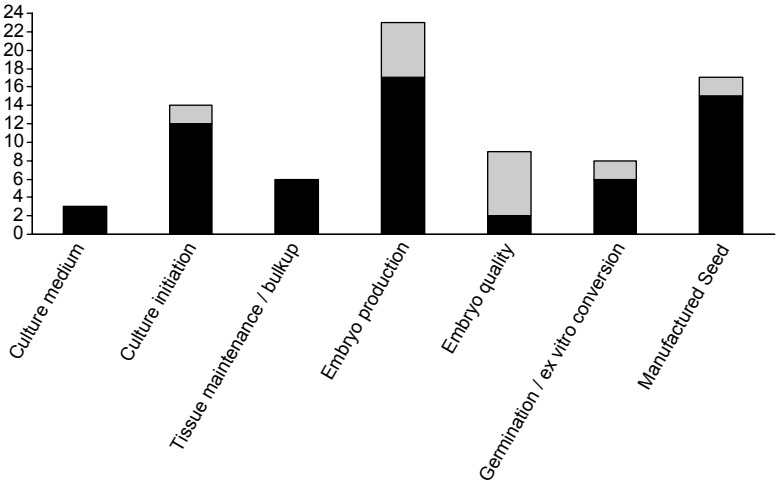

FIG. 1. US patents issued and applications published since 1990 on conifer SE technology by step or subject matter. Distribution of 80 Conifer SE U.S. Patents by Subject Matter: ■ Issued; ▨ Application.

TABLE 1

CONIFER SPECIES TRANSFORMED AND REGENERATED

Species	Method	Explant	Selectable marker	Reference
Picea glauca	Biolistics	EC	nptII	Ellis et al., 1993
	A. tumefaciens	EC	nptII	Klimaszewska et al., 2001b
Picea abies	Biolistics	EC	nptII	Walter et al., 1999
	Biolistics	EC	bar	Clapham et al., 2000
	A. tumefaciens	EC	nptII	Klimaszewska et al., 2001b
Picea mariana	Biolistics	EC	nptII	Charest et al., 1996
	A. tumefaciens	EC	nptII	Klimaszewska et al., 2001b
Pinus radiata	Biolistics	EC	nptII	Walter et al., 1998
	A. tumefaciens	EC	nptII	Arce-Johnson et al., 2002
	A. tumefaciens	MZE, SA	nptII	Charity et al., 2002
	A. tumefaciens	EC	nptII	Charity et al., 2005
	A. tumefaciens	MZE	nptII	Grant et al., 2004
Pinus taeda	Biolistics	EC	nptII	Kodrzycki et al., 2002
	Biolistics	MZE	nptII	Tang and Samuels, 2002
	A. tumefaciens	MZE	hpt	Tang et al., 2001
	A. tumefaciens	EC	nptII	Connett et al., 2002, 2003
	A. tumefaciens	SA	nptII	Gould et al., 2002
	A. tumefaciens	MZE	hpt	Tang, 2003
Pinus pinaster	Biolistics	EC	hpt	Trontin et al., 2002
	A. tumefaciens	EC	hpt	Trontin et al., 2002
Pinus strobus	*A. tumefaciens*	EC	nptII	Levee et al., 1999
Pinus sylvestris	Biolistics	Pollen	nptII	Aronen et al., 2003
Larix laricina	Biolistics	EC	nptII	Klimaszewska et al., 1997
Larix kaempferi × *decidua*	*A. tumefaciens*	EC	nptII	Levee et al., 1997

EC, embryogenic callus; MZE, mature zygotic embryos; SA, shoot apex; nptII, neomycin phosphotransferase; hpt, hygromycin phosphotransferase; *bar*, phosphinothricin acetyl transferase.

infection, and effective selection strategies have contributed to the success of genetic transformation in conifers. Among the commercially important conifers, hybrid larch was the first to be stably transformed via co-cultivation of embryogenic tissue with *A. tumefaciens* (Levee et al., 1997). Subsequently, this approach was successfully applied to several species of spruce (Klimaszewska et al., 2001b). In this study, stably transformed plants were regenerated for all spruce species tested, but transformation efficiency varied among species. The highest transformation efficiency was obtained for *Picea mariana*, followed by *P. glauca* and *P. abies*. The detailed molecular characterization of translines regenerated in this study revealed that the majority of *P. mariana* translines integrated multiple copies of the transgenes, whereas mostly single copy integrations were detected in the translines of the other two species (Klimaszewska et al., 2003). These results are significant from the standpoint that higher transformation efficiency does not always result in production of transgenic events that would be desirable in a commercial product. This highlights the importance of finding a balance between transformation efficiency and the quality of transgenic events necessary for successful commercialization.

Several reports have appeared in the past five years on *Agrobacterium*-mediated transformation of radiata pine germplasm grown in Chile (Arce-Johnson et al., 2002) and New Zealand (Charity et al., 2002, 2005; Grant et al., 2004). *Agrobacterium*-mediated transformation protocols have also been reported for diverse germplasm of loblolly pine grown in the southeastern US (Tang et al., 2001; Gould et al., 2002; Connett et al., 2003; Tang and Tian, 2003;). For both species, embryogenic tissue established from immature zygotic embryos, as well as organogenic cultures obtained from shoot apices, cotyledons, and mature zygotic embryos, have been used as explants for inoculation with *A. tumefaciens* (Table 1). Potential advantages of shoot apices or mature cotyledons as target tissues for transformation are that these explants are available year round, can be stored inexpensively, and allow transformation of a large number of genotypes. However, because of low transformation efficiency, increased risk of chimeric plants associated with organogenic regeneration and production of very few transgenic plants from a single event (Tang et al., 2001), the transformation of such explants is better suited to the evaluation of candidate gene functions than the large-scale production of translines required for final selection of a line that meets all the criteria required for commercialization. On the other hand, methods that employ embryogenic tissue as an explant source (Connett et al., 2002; Charity et al., 2005) are now applicable to a wide range of genotypes and suitable for high-throughput gene testing as well as for production of millions of copies of the selected transgenic events for commercial scale planting.

The success of any transformation system depends on the efficient delivery and expression of the selectable marker gene in the cells that are capable of regeneration into plants. A review of conifer transformation reveals that neomycin phosphotrasferase II (nptII), which confers resistance to aminoglycoside antibiotics (kanamycin and geneticin), is the most commonly used selectable marker gene for different species (Table 1). The hygromycin phosphotransferase gene (*hpt*) that confers resistance to hygromycin has also been used for transformation of loblolly (Tang et al., 2001; Tang and Tian, 2003) and maritime pine (Trontin et al., 2002).

However, there is only one report (Clapham et al., 2000) where an herbicide resistant gene (*bar*) has been used as a selectable marker. The use of positive selectable marker genes (Haldrup et al., 1998) and marker removal strategies suitable for asexually propagated plants (Schaart et al., 2004) has not yet been published for conifer species. This highlights the need for concerted research efforts for marker development for conifers.

It is evident that the majority of conifer species are now amenable to transformation at an operational level. The development of efficient *Agrobacterium*-based transformation systems has enabled the establishment of an increasing number of field tests for evaluation of genes of interest in various conifer species (van Frankenhuyzen and Beardmore, 2004). The transformation challenge, therefore, lies in extending these protocols to elite genotypes and also manipulating the transformation parameters to increase the proportion of events with a single insert integration pattern that is desirable in commercial products. The feasibility of commercial scale transformation and discovery of new genes have, most recently, sparked the industrial interest in developing and commercializing transgenic conifers with value-added traits.

Hardwood Tissue Culture and Transformation

Today, hardwoods represent approximately 40% of the forest plantations in the world (FAO, 2000), a significant proportion of which are clonally propagated. Breeding and clonal programs provide elite germplasm for commercial transformation with value-added traits, as well as highly transformable or early flowering genotypes for gene discovery research. An efficient means of regenerating trees from transgenic cells is critical for the transformation of hardwoods. Tissue culture systems based on organogenesis and SE have been developed for many hardwood species. Organogenesis is the regeneration system used predominantly in studies with *Populus* species or hybrids, and all reported transformations were done through organogenesis-based regeneration (reviewed in Confalonieri et al., 2003; Dai et al., 2003). Similarly, organogenesis systems were used in transformation studies of acacia (Xie and Hong, 2002), black locust (Igasaki et al., 2000), silver birch (Wang et al., 2001), English elm (Gartland et al., 2000), sweet orange and citrange (Yu et al., 2002), sweetgum (Kim et al., 1999), and several *Eucalyptus* species and hybrids (MacRae and van Staden, 2000; Tournier et al., 2003; Deng et al., 2004). Many recent reports present the use of SE systems for transformation. Transgenic plants were recovered for English walnut (Escobar et al., 2000), European chestnut (Corredoira et al., 2004), rubber tree (Jayashree et al., 2003), and sweetgum (Merkle et al., 2003). Meanwhile, SE systems have also been developed or improved for *Eucalyptus globulus* (Nugent et al., 2001; Pinto et al., 2002), *E. pellita* (Xie and Chen, 2001), hybrid sweetgum (Vendrame et al., 2001; Merkle et al., 2004), and hybrid *Liriodendron* (Merkle et al., 2004). A significant development recently has been the establishment of organogenesis systems from mature genotypes of sweetgum (Merkle and Battle, 2000; Merkle et al., 2003), Holm oak (Mauri and Manzanera, 2004), and English oak (Toribio et al., 2004). It is foreseeable that these systems will not only be used for clonal propagation, but also for transformation. In addition to SE and organogenesis-based transformations, axillary buds of *E. globulus* were infected with *Agrobacterium* with the assistance of sonication and vacuum infiltration and high-efficiency stable transformation was obtained (Gallego et al., 2002). This system showed great potential of transforming clones without going through a dedifferentiation process, which tends to be a limiting factor for many elite species and clones.

A broader range of selectable markers has been developed for hardwoods than for conifers. There are several recent examples in which the transgene for the introduced trait also served as a selectable marker. These include herbicide tolerance genes such as the glyphosate resistance gene *CP4* (Meilan et al., 2002), *bar* (phosphinothricin acetyl transferase; Confalonieri et al., 2000; Harcourt et al., 2000), and *crs-1* (mutant form of acetolactase synthase; Confalonieri et al., 2003), and a heavy metal detoxifying gene *merA* (mercury reductase; Rugh et al., 1998; Che et al., 2003). A procedure for selectable marker removal has also been developed and demonstrated with hybrid poplar (reviewed in Ebinuma et al., 2001).

Despite the progress in recent years, challenges remain for the transformation of hardwood trees. Although elite individuals of several hardwoods such as poplars, eucalypts, and sweetgum can be maintained through vegetative propagation methods, genotypic variation in regeneration prohibits the capture of many genotypes in tree improvement programs. Even for the elite genotypes that are propagated for clonal deployment, transformation can be limited by *Agrobacterium* susceptibility or *in vitro* regeneration. At the moment, hardwood transformation is largely limited to either the clones that are easy to transform and regenerate or juvenile materials that have higher regeneration and transformation potential. For example, reliable transformation systems have been developed for many species or hybrids belonging to the more amenable taxonomic Populus sections, whereas there are very few reliable transformation systems for species or hybrids in the Tacamahaca or Aigeiros sections (e.g. *Populus deltoides*; reviewed by Confalonieri et al., 2003). Maturation is another common problem. While seedling or juvenile tree explants are relatively easy to regenerate and transform, explants from mature trees tend to lose regeneration potential. Unfortunately, elite clones are selected from older trees with years of field performance data. It is encouraging to see that mature sweetgum genotypes can be regenerated through SE using inflorescence tissues (Merkle and Battle, 2000; Merkle et al., 2003), but the maturation challenge remains for many other hardwood species or hybrids.

To successfully conduct tree biotechnology, it will also be necessary to be able to introduce multiple transgenes and maintain the expression of transgenes for multiple years. It has been recently demonstrated that multiple transgenes can be delivered either through stacking them on the same construct (Harcourt et al., 2000; Fan et al., 2002) or through co-transformation (Li et al., 2003a). In cases where transgenic poplars in multiyear field tests have been examined (Meilan et al., 2002; Pilate et al., 2002), the phenotypes conferred by the transgenes were stable over time.

Tree Genomics and Gene Discovery

It is convenient to separate DNA-based biotechnology into candidate gene discovery (cDNA and gene sequencing; microarrays and other expression analyses; markers, gene mapping, and association genomics) and functional testing (transgenics). Gene discovery helps uncover information that can be used in breeding and genetic engineering of trees for faster growth, disease

resistance, tolerance to abiotic stress, and improved wood characteristics. In some cases, sequence comparisons with model species may identify genes that are likely to be involved in producing a valuable trait. Although *Arabidopsis* makes little secondary xylem, many important advances in understanding related to wood development have occurred in this model organism, including the first proof of cloning of a plant cellulose synthase through complementation of a mutant (Arioli et al., 1998) and identification of transcription factors that regulate vascular development (for recent examples, see Prigge et al., 2005; Sawa et al., 2005). Thus, the work on model plant species has provided an invaluable jump-start to the molecular characterization of wood development and tree growth. Protocols have been developed for manipulating *Arabidopsis* into production of secondary xylem (Chaffey et al., 2002; Ko et al., 2004), and it is certain that *Arabidopsis* and crop species will continue to inform research on trees. However, herbaceous models can only go so far in elucidating some aspects of tree biology, such as wood development. One practical reason for this is that it will be very difficult to collect the gram quantities of vascular cambium from *Arabidopsis* that are required for some molecular approaches. In addition, researchers studying tree–pathogen interactions will wish to work with the actual species. Conifers have been separated from angiosperms for at least 250 million years, so sequence divergence makes it difficult to be sure that related genes from pine and *Arabidopsis* have the same function. To overcome these obstacles, a common approach has been to use the data obtained from sequencing projects on tree species to design microarrays for genome-wide studies of gene expression. The patterns of gene expression in different tissues or under different environmental stimuli lead to the identification of candidate genes, which can then be targeted for in-depth analysis, either to find natural variants or to alter their expression through transgenic technologies.

Gene Discovery in Hardwoods

Characterization of genomes is under way for a variety of forest tree species. A major challenge in providing a summary of the status of genomics in tree species is that current progress is so rapid; a number of projects have not been described in publications, and up-to-date documentation is often limited to the Internet. Table 2 lists a number of websites related to genomics and gene discovery in trees. Hardwood (angiosperm) species that belong to genus *Populus*, especially *P. trichocarpa* (black cottonwood), dominate tree molecular biology by virtue of a recently released genome sequence. Brunner et al. (2004) reviewed the value of *Populus* as a model for forest biotechnology and outlined the status of the sequencing project in 2004. The US Department of Energy Joint Genome Institute and the International Poplar Genome Consortium sequenced the cottonwood genome to an average coverage of 8-fold. A first draft assembly was generated, which can be accessed on the web (Table 2). The random nature of the sequencing left thousands of sequence gaps remaining, but it can be estimated that at least 90% of the genes in the genome were represented in this collection of sequences. An updated version with annotations will likely be released before publication of this review. Gene sequences, expressed sequence tags (ESTs), and full-length cDNA sequences from other members of the genus, including aspens such as *Populus tremula* and its hybrids, are being generated by groups worldwide, resulting in over 270 000 *Populus* sequences currently stored at the

TABLE 2

WEBSITES RELATED TO TREE GENOMICS

Category/Species	Website
General	
Dendrome	dendrome.ucdavis.edu
METLA	www.metla.fi/info/vlib/forestgen/
Plant Genome Database	www.plantgdb.org
Populus	
Arborea	www.arborea.ulaval.ca/en/what/description.php
JGI/International Populus Genome Consortium	genome.jgi-psf.org/Poptr1/Poptr1.home.html
SweTree Technologies	www.swetreegenomics.se/default.asp?pageid = 2096&path = 4040%2C4041
Treenomix	www.treenomix.com
Eucalyptus	
International Eucalyptus Genome Consortium	www.ieugc.up.ac.za
Genolyptus	genolyptus.ucb.br/genolyptus-english.jsp
Pinus	
Functional Genomics	www.fungen.org/Projects/Pine/Pine.htm
Loblolly Pine Genome Project	dendrome.ucdavis.edu/lpgp
Loblolly Xylem ESTs	pinetree.ccgb.umn.edu
Picea	
Arborea	www.arborea.ulaval.ca/en/what/description.php
Treenomix	www.treenomix.com
Other	
Acacia	www.ffp.csiro.au/tigr/molecular/acacia.html
Citrus (Brazil)	200.144.120.131/PHP/html/modules/home/html/intro2.htm
Citrus (Spain)	citrusgenomics.ibmcp-ivia.upv.es/about_cfgp/summary.shtml
Malus	titan.biotec.uiuc.edu/apple/apple.shtml
Medicago	www.medicago.org/genome/about.php
Prunus	www.genome.clemson.edu/gdr/projects/prunus/unigene/

National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov). A particularly large set has been accumulated by Sandberg and co-workers at the Umea Plant Science Center (Sterky et al., 2004). Other EST sets include developing xylem (Dejardin et al., 2004) and stress-induced leaves (Nanjo et al., 2004). Bohlmann and colleagues at the University of British Columbia have contributed more than 20% of the publicly accessible sequences, but no publication is yet available for citation. The cottonwood genome sequence is an important advance for all of plant molecular genetics, not just forestry. Because *Populus* is evolutionarily relatively close to *Arabidopsis* (much closer than rice, the other completely sequenced plant), comparisons between the two genomes are likely to be as informative as the comparison of the human and mouse genomes (Mouse Genome Sequencing Consortium, 2002) has been for mammalian molecular biology. As demonstrated by Blázquez and Weigel (2000) for the *LEAFY* gene, comparison of *Arabidopsis* and cottonwood sequences can reveal conserved functional elements of genes such as regulatory sequences in promoters.

Several microarrays have been produced in *Populus*, although many results are not yet published. Sandberg and coworkers examined wood formation in *P. tremula* × *Populus tremuloides* with a microarray representing almost 3000 genes (Herzberg et al., 2001) and then studied the cambial region in more detail with a microarray representing approximately 13 000 genes (Schrader et al., 2004). By finely sectioning the developing wood to separate the stages of phloem and xylem formation, they were able to detect variations in expression of many genes as the cells divided, expanded, and lignified. The patterns of expression of a number of enzymes involved in cell proliferation and cell wall formation were found to be more strongly expressed at the expected stages, providing confidence that the changes in expression patterns found for transcription factors and other genes whose functions are not precisely known were meaningful.

Eucalyptus is another hardwood of great economic and research importance in forestry, although gene sequencing has not progressed as far as in *Populus*. There is a loose international association of academic and industry scientists interested in *Eucalyptus* DNA markers and gene sequences, the *Eucalyptus* Genome Initiative, and a large sequencing project for *Eucalyptus*, Genolyptus, was initiated in Brazil with funding from a combination of government and commercial sources (Table 2). The Kazusa Institute and Oji Paper Company issued a press release during 2004 in the newspaper *Mainichi Shimbun* that a separate genome sequencing project for *Eucalyptus* was under way (www.business-support-chiba.jp/cgi-bin/dire/backnumber.cgi?act = 5). An EST project in *Eucalyptus grandis* has accumulated more than 170 000 sequences from 20 libraries (Strabala, 2004). In none of these cases have the data been publicly released. The NCBI lists approximately 2600 sequences for *E. grandis* and a further 1800 for other members of the genus. Two EST-based studies of wood formation in *Eucalyptus* have been published. Kirst et al. (2004) identified a negative correlation between growth and lignin biosynthesis in *E. globulus* × *E. grandis* backcrossed to *E. grandis*. Gene expression levels for approximately 2000 genes were characterized in 91 individuals using a microarray. After ranking the results by diameter, it was found that multiple genes involved in the synthesis of lignin monomers were less expressed in the larger trees. In a library enriched for xylem-expressed sequences, Paux et al. (2004) identified about 170 genes upregulated during xylem development in *E. gunnii*.

Although birch (*Betula* spp.) has been the object of some molecular and transgenic studies (e.g., Lemmetyinen et al., 2004), there are fewer than 3000 sequences from this genus listed at NCBI. Likewise, *Acacia* (under 600 entries) and teak (*Tectona*, about 80 entries) are apparently not undergoing molecular characterization to any large extent. Of peripheral relevance to forestry are several genera of fruit trees that are the object of EST sequencing projects: *Citrus*, *Malus*, and *Prunus* (Table 2). Although much research on these species is related to fruit quality and organism-specific disease resistance, it is likely that the sum of the information produced will advance understanding of forest species. Additionally, an international sequencing project for *Medicago truncatula*, which began about 4 yr ago (Bell et al., 2001), is well under way. Although this is a herbaceous legume, its gene sequences and genome organization are likely to be very useful to understanding *Acacia* and other trees from the same family. Further information about the project, scheduled for completion in 2006–2007, can be obtained at their website (Table 2).

An additional approach to identifying genes and their functions is to create mutants by insertion of transgenic DNA. The method known as 'activation tagging' creates dominant mutations that result in misexpression or overexpression of native genes (Weigel et al., 2000). A trial of over 600 transgenic lines of activation-tagged poplars has produced multiple novel phenotypes, including dwarfed stature mediated by changes in gibberellin metabolism (Busov et al., 2003). A related method links a reporter gene to random poplar promoters and enhancers, providing a means of identifying genes that are expressed in xylem (Groover et al., 2004).

Gene Discovery in Conifers

For conifers, with genomes that are far larger than those of the completed model species and which contain large proportions of repetitive DNA, full genome sequences are currently out of the realm of feasibility. Instead, cDNA sequencing projects have been the major form of gene characterization. The genera *Pinus* and *Picea* are most heavily studied, in particular loblolly pine (*P. taeda*), which is a highly valued timber source in the southern US, and white spruce (*P. glauca*), grown in the northern US and Canada. For loblolly pine, more than 180 000 DNA sequences can be accessed through NCBI. The sequences were derived mostly from projects at North Carolina State University and the University of Georgia. The earliest extensive sequencing of loblolly focused on cDNA libraries from whole seedlings (Kinlaw et al., 1996) and xylem (Allona et al., 1998; Whetten et al., 2001). ESTs from pine embryos have been collected for the purpose of improving SE (Ciavatta et al., 2001). About 22 000 sequences from other pines are stored at NCBI, primarily from *Pinus pinaster* (19 000 sequences) plus over 1000 from *P. radiata*. *P. radiata* has also been the subject of a large EST characterization effort that has not yet been made public, where over 340 000 ESTs were obtained from 40 separate cDNA libraries (Strabala, 2004).

Despite the daunting size of the pine genome, about 25 pg or more than 2.5×10^{10} bp (Bogunic et al., 2003), there is still potential for a sequencing project that is restricted to the gene-rich regions of the chromosomes. The nascent Loblolly Pine Genome Project goals are outlined at the website listed in Table 2. Two

technologies have been developed for enriching genes out of a genome filled with nontranscribed repetitive sequences. Methyl filtration relies on using a bacterial restriction system to select against sequences that are methylated (usually repetitive elements) during the cloning prior to sequencing (Rabinowicz et al., 1999). High-C_0t selection takes advantage of the kinetics of annealing of complementary strands of DNA to select out repetitive sequences (Peterson et al., 2002). These methods have already been applied to the maize genome with encouraging results (Fu et al., 2004), and it is likely that they will continue to be refined and applied to the loblolly pine genome.

Establishment of microarrays for loblolly pine is also less advanced than for *Populus*. Morse et al. (2004) described a small array of *c*. 300 pine cDNAs being used to study response to pathogen infection. An array of approximately 2000 loblolly pine cDNAs was used to study changes in gene expression during drought stress (Watkinson et al., 2003). Adventitious root development in *Pinus contorta* was studied using a similar array (Brinker et al., 2004). An alternative means of analyzing gene expression is serial analysis of gene expression (SAGE). This method relies on the generation of many short DNA fragments from a pool of RNA, which are then sequenced. The frequency of a given sequence appearing provides an estimate of transcript abundance, and by matching each sequence with the corresponding gene, it is possible to learn the changes in expression for many genes simultaneously. SAGE was applied to lignifying loblolly xylem and changes in gene expression were noted (Lorenz and Dean, 2002), but most of the SAGE tags could not be linked to a gene. Both microarrays and SAGE will benefit from more extensive knowledge of the pine gene sequences.

For spruce, the majority of the available sequences are from *P. glauca* (55 000 sequences deposited at NCBI) and *Picea sitchensis* and hybrids (24 000 deposited sequences, combined). A spruce unigene set of approximately 20 000 sequences has been developed from nearly 35 000 ESTs (Rungis et al., 2004). *P. glauca* somatic embryo development was studied using a 2000-gene loblolly pine microarray (Stasolla et al. 2004), showing that treatment with PEG induced a number of regulatory genes involved in embryogenesis. A small ($<$400 genes) array of *P. abies* cDNAs was used to characterize embryogenic cultures in Norway spruce (van Zyl et al., 2003).

DNA Markers for Mapping and Candidate Gene Identification

Because breeding of forest trees is such a long process, information about specific genes or alleles that are associated with improved wood yield or quality have the potential to be highly valuable if the cycle can be shortened or the size of progeny tests can be decreased. Thus, DNA markers have been in development in forest species for more than 10 years (reviewed in Kumar and Fladung, 2004). An important consequence of the declining costs of sequencing has been the wealth of DNA markers obtained, particularly simple sequence repeats (SSRs; also known as microsatellites) and single nucleotide polymorphisms (SNPs). These markers are generally applicable over a wide range of genotypes and are frequently conserved among species of the same genus. In spruce, 25 SSR markers were obtained from approximately 35 000 ESTs (Rungis et al., 2004). SNPs are much more frequent: a collection of fewer than 20 000 ESTs from *P. pinaster* produced over 1000 candidate SNPs (Dantec et al. 2004). In cases such as a quantitative trait nucleotide (QTN), the sequence changes can be the direct cause of an important phenotype, but more commonly, they are genetically linked to as-yet-unidentified genes. Gill et al. (2003) found a sequence change in the loblolly cinnamyl alcohol dehydrogenase (CAD) gene that produced a null allele that is directly responsible for altered lignin content and reddish-brown wood in homozygous trees (MacKay et al., 1997).

Because of the interest in linking markers with quantitative trait loci (QTLs) and because measurement of the traits is usually a multi-year process, most mapping studies in trees have been confined to individual families. During the past year, examples were published for *P. abies* (Achere et al., 2004), *P. radiata* (Devey et al., 2004), and Douglas-fir (Wheeler et al., 2005). However, with the accumulating gene sequence data for loblolly has come the ability to provide a map that is informative for other members of the genus *Pinus* (Komulainen et al., 2003) and even provide links between the chromosomes of different genera within *Pinaceae* (Krutovsky et al., 2004). Such a map provides a scaffold upon which to build a detailed model of the pine genome, should a loblolly pine genome sequencing effort be successful. Although it may not be possible to establish the precise arrangement of genes along the pine chromosomes, a marker map will aid in putting 'bins' of closely linked genes in useful order.

An additional use of SNPs is in association genetics (Neale and Savolainen, 2004). This method takes advantage of the natural variation of phenotype and gene sequence in a species and also of the many recombination events that occurred over recent millennia. These recombinations produced very low linkage disequilibrium in trees; within a population the amount of flanking DNA associated with a particular allele averages only a few thousand bases (Brown et al., 2004). Therefore, if a SNP in a candidate gene is frequently associated with a phenotype during a screen of a population of unrelated trees, there is a very good chance that the SNP is in a gene that contributes to the phenotype. The genomics programs in *Populus*, *Eucalyptus*, *Pinus*, and *Picea* have generated a wealth of candidate genes suitable for association genetics.

Potential Products from Transgenic Trees

The large body of genomics research outlined above has identified many potential growth and developmental genes, but it is still highly exploratory. The genes that show significant changes in microarray experiments or have *Arabidopsis* homologs with interesting mutations are only candidates until their function is defined. Study of transgenic plants will provide vital evidence for the functions of genes of interest but, for the most part, those results will come a fair distance in the future. There are, however, already numerous examples where significant data have been collected indicating how a specific gene can be used to alter tree growth and wood development in commercially valuable ways. Although some very important advances have occurred in conifers, work with *Populus* species dominates this field because poplars are generally easier to transform.

The targets for forest product development can be divided broadly into product quality traits and agronomic traits. Agronomic traits are those important to productivity and silvicultural issues such as growth rates and stress or insect tolerance, while product quality traits are more concerned with an improved end use of the

material produced such as alterations in lignin content and strength of wood. For this discussion, wood yield obtained through improved growth and biomass will be considered as a product quality trait, as it affects the end use processes such as pulp and paper making. Agronomic traits such as herbicide tolerance and insect resistance have been demonstrated for multiple crop plants, and it is relatively easy to introduce these traits into trees for evaluation of performance.

Product Quality Traits

The tree research community can readily build on the research done on agricultural crops for agronomic traits. However, quality traits for trees are quite different from quality traits being developed for most agricultural crops. Tree quality traits relate primarily to tree form and wood, and fiber properties that make the wood derived from trees more valuable as a timber product, as a feedstock for pulp mills and timber mills, for furniture manufacture, or as a source of chemical cellulose and specialty chemical products. The development of these quality products will be dependent on research that is specifically focused on understanding the molecular basis for tree biology.

Wood modification. A great deal of research has been done on the understanding of cell wall formation (Mellerowicz et al., 2001; Somerville et al., 2004) and also the process of wood formation (Plomion et al., 2001). The modification of lignin is a focus given the possibilities of affecting end products through its manipulation (Baucher et al., 2003). It had been known for some years that change in expression of certain enzymes in the lignin biosynthetic pathway results in alterations of lignin composition and easier removal of lignin. These include CAD (Baucher et al., 1996; Lapierre et al., 1999) and 4-coumarate ligase (4CL; Hu et al., 1999), which were tested in transgenic poplar.

When aspen trees were produced that combined 4CL down-regulation with cinnamaldehyde 5-hydroxylase (Cald5H) over-expression, an increased syringyl to guaiacyl (S/G) ratio was added to the previously demonstrated lignin reduction and cellulose increase (Li et al., 2003b). These effects, especially in combination, are beneficial to kraft pulping applications.

The ferulate-5-hydroxylase (F5H) enzyme was shown to be a rate-limiting step in the production of syringyl units in *Arabidopsis*, and overexpression of the gene encoding this enzyme using the cinnamate-4-hydroxylase (C4H) promoter to drive expression specifically in cells involved in the lignin biosynthetic pathway significantly increased the percentage of syringyl lignin. Analysis of poplar transformed with a cinnamate-4-hydroxylase:ferulate-5-hydroxylase construct demonstrated significant increases in pulping efficiency from 2-yr-old greenhouse-grown trees (Huntley et al., 2003).

Genes other than those in the lignin biosynthetic pathway have also been used to alter wood composition. Reduction in laccase through antisense lac3S expression in transgenic poplar has led to an alteration of the phenolic content and aberrations in xylem fiber structure without altering lignin content or composition (Ranocha et al., 2002). Antisense suppression of the peroxidase gene *prxA3a* in aspen resulted in lower lignin content and a higher syringyl/vanillin ratio because of reduced vanillin units (Li et al., 2003b).

Wood yield through increased growth. Increasing tree growth rates has been a primary goal of tree improvement through breeding programs and advances in silvicultural practices, and is a natural target for improvement through genetic modification. Mapping studies have identified a number of genes that may be involved in control of growth rates but there has been little progress in comprehensive testing of these candidate genes.

It has been reported that a gene that affects lignin biosynthesis can also affect growth. Downregulation of 4-coumarate ligase (4CL) in aspen leads to reduction of lignin levels, increased cellulose and increased biomass accumulation (Hu et al., 1999). Interestingly, a gene that affects cellulose biosynthesis has also been reported to affect tree growth. Biomass was increased by introducing a bacterial cellulose binding domain gene into poplar (Levy et al., 2002).

In addition to the growth gains related to cell wall pathway modifications described above, there have been a limited number of genes tested that address growth rates directly. Overexpression of glutamine synthetase in poplar has led to large increases in height growth in multi-year field tests where increases of 41% over controls were observed after the third year of growth (Ping Jing et al., 2004). These same transgenic poplar plants had shown increases in growth over controls in greenhouse experiments of up to 76% after 2 mo. and 21.3% after 6 mo. (Gallardo et al., 1999). This variability in performance of transgenic trees between the greenhouse and field environments is an important consideration when making predictions of potential agronomic benefits.

Gains in biomass accumulation have also been achieved through alteration in gibberellin metabolism. Overexpression of a gibberellin 20-oxidase gene in hybrid aspen led to 20-fold higher active gibberellin levels and increased dry shoot biomass 64% over controls (Eriksson et al., 2000). Notably, the increase in stem dry biomass was 126% over the controls. The GA 20-oxidase overexpression also resulted in longer internodes and longer xylem fibers. However, the transgenic plants were deficient in rooting when transferred to soil as compared with control plants. This growth enhancement in hybrid aspen in response to increasing gibberellin concentrations appears to be transgene specific. Overexpression of a giberellin 3-oxidase in hybrid aspen led to increases in gibberellin activity but no major morphological differences (Israelsson et al., 2004). This study showed that levels of GA_4, not GA_1, were linked to differences in internode length. Overexpression of another gene involved in the metabolism of this group of compounds, GA 2-oxidase, through activation tagging showed the importance of gibberellin levels in regulating tree height (Busov et al., 2003). Elevated levels of GA 2-oxidase led to a lower level of GA_1 and GA_4 and dramatically reduced internode length that could be rescued by the exogenous application of GA_3. Overexpression of the *Agrobacterium* cytokinin beta-glucosidase *rolC* gene has an effect on vegetative developmental while altering the levels of indole-3-acetic acid, cytokinins, and gibberellins (Nilsson et al., 1996). Reduced apical dominance of the hybrid aspen plants was correlated with reduced gibberellin and indole-3-acetic acid while cytokinin levels were increased. Stem fasciation was also observed in the transgenic trees.

Agronomic Traits

A transgenic approach has also proven to be useful in imparting biotic and abiotic stress resistance to forest trees. Pathogen and pest control in tree plantations is relatively difficult and costly owing to the size of the trees, the perennial nature of the plantation, and the

plantation size. In addition, some plantation species are limited in their distribution and productivity owing to drought and cold sensitivity. Improvements in genetic resistance to stress would allow for greater flexibility in the species and geographies that are amenable to sustainable plantation forestry. Recent examples of resistance to stress from insect feeding, fungal infection, herbicide treatment, drought treatment, and cold tolerance in loblolly pine and *Populus* are described below.

Biotic stress resistance. Insect tolerance is a very important trait for ensuring healthy and productive conifer and hardwood plantation forests as insect predation lowers yields through direct damage and through secondary pathogen infection. Recently, it was shown that expression of a *Bacillus thuringiensis* (BT) toxin gene in loblolly pine plantlets led to increased resistance against pine caterpillar (*Dendrolimus punctatus* Walker) in insect feeding bioassays (Tang and Tian, 2003). The level of the BT toxin expression was directly related to the degree of insect resistance observed for most of the transgenic lines analyzed. *Populus* transformed with a polyphenol oxidase gene showed increased resistance to forest tent caterpillar feeding in leaf disk assays (Wang and Constabel, 2004). The larvae in this assay experienced higher mortality and reduced average weight gain when feeding on the transgenic material compared with controls.

Resistance to *Septoria* fungal inoculation in leaf disk assays was observed in poplars overexpressing an oxalate oxidase gene (Liang et al., 2001). In transgenic aspen expressing a pinosylvin synthase gene led to increased resistance to the fungus *Phenllinus tremulae* in selected transgenic lines (Seppanen et al., 2004). Interestingly, transgene expression was not always directly correlated to the rate of wood decay following fungal infection.

Weed pressure severely impacts the establishment of a young tree plantation. Herbicide resistance acts in this case to increase growth and yield by limiting weed competition in the first few years of growth. Resistance to the herbicide Roundup® was demonstrated in field tests of transgenic poplars (Meilan et al., 2002). Two-year field studies showed that 10% of the transgenic lines were resistant to foliar damage following application of commercial rates of the herbicide; the growth rates of these lines were also unaffected. This study showed that performance of transgenic poplars with the *CP4* EPSP synthase gene alone was superior to that of trees with both the *CP4* gene and the *GOX* glyphosate oxidase gene.

Abiotic stress resistance. Plant regulatory networks of gene expression in drought and cold stress responses have recently been reviewed (Chinnusamy et al., 2004; Zhang et al., 2004). The *Arabidopsis* CBF (C-repeat binding factor) family of transcription factors is well known for regulating cold-responsive gene expression (Jaglo-Ottosen et al., 1998; Liu et al., 1998; Kasuga et al., 1999). The *Arabidopsis* CBF1 (*At*CBF1) cDNA was ectopically expressed in hybrid aspen (*P. tremula* × *tremuloides*, clone 717), and the resulting transgenic hybrid aspen was more freeze-tolerant than the wild type, both before and after cold acclimation (Benedict et al., 2004). Transcript profiling using a 13 000-gene *Populus* microarray demonstrated that *c*. 4% of genes upregulated at the seventh day of low-temperature exposure in the wild-type trees were also upregulated at warm temperature in the *At*CBF1-expressing transgenic trees. The promoter regions of 35 of the 64 *At*CBF1-upregulated *Populus* genes contained at least one potential CBF binding site (Benedict et al., 2004). Puhakainen et al. (2004) isolated a silver birch dehydrin gene, *Bplti36*, which was highly induced by cold, drought, and ABA treatment. The promoter regions of *Bplti36* contain five CBF binding sites and one ABA response element. It was found that the *Bplti36* promoter activity was highly induced in the *At*CBF3-expressing *Arabidopsis* at warm temperatures. The above results strongly suggested that the CBF regulatory pathways are also present in trees and are highly conserved in flowering plants.

Poplars transformed with a pine glutamine synthetase gene were found to have increased drought resistance (El-Khatib et al., 2004). Analysis of these plants indicated increased net photosynthesis regardless of drought treatment but an increase in stomatal conductance in the transgenic plants was only apparent before drought treatment. The higher net photosynthesis of these plants may also be related to improved growth performance in field tests described above (Gallardo et al., 1999; Ping Jing et al., 2004).

Regulatory Considerations for Transgenic Trees

Field testing and approval for large-scale plantings for transgenic trees in the US are governed by several federal regulatory agencies. In 1986, the US developed the Coordinated Framework for Regulation of Biotechnology. A key aspect of the framework was the understanding that the characteristics, composition and intended use of the GM product were important considerations when regulating them, not the methods by which they were developed. Under this framework, the United States Department of Agriculture, Animal and Plant Health Inspection Service (USDA APHIS), Food and Drug Administration (FDA), and Environmental Protection Agency (EPA) coordinate the regulation of genetically modified (GM) crop products. The role and *modus operandi* of each agency in assessing the safety and approval of GM products depends on whether the product is intended for food and feed (FDA) or insect pest (EPA) management (Re et al., 1996).

Among the US agencies, APHIS plays the major role in overseeing field tests and eventual deregulation of GM plants. The US system for oversight and regulation of GM plants has worked effectively for over 20 years to ensure the safety of these products and protection of environment. Over 60 products have been given deregulated status, and many of these have been widely adopted by farmers all over the country. APHIS' current authorization comes under the Plant Protection Act of 2000, which incorporates earlier laws and provides the authority to regulate plants, plant products, certain biological control organisms, noxious weeds, and plant pests. APHIS conducts in-depth analyses as part of the permitting process and review of petitions for nonregulated status in fulfillment of its obligations under the National Environmental Policy Act (NEPA). In 2002, APHIS created the Biotechnology Regulatory Services unit within the Agency that now manages all its activities with GM organisms (www.aphis.usda.gov/brs/). Many other countries of the world, including Canada (www.inspection.gc.ca/english/sci/biotech/reg/bare_shtml) and Europe (www. europa. eu.int/comm./food/biotechnology/index_en.htm), have developed similar stringent regulations for testing and release of GM products. Several developing countries such as China and Brazil are also in the process of developing effective infrastructure for regulation of GM crops and trees (Strauss, 2003).

There is not yet, with the exception of *Bt* poplar in China (Hu et al., 2001), any large-scale plantings of transgenic forest trees. However, field tests are currently being conducted in several countries, with the

majority of these field tests occurring in the US (van Frankenhuyzen and Beardmore 2004) in the past 5–7 yr. The majority of these trials have been conducted under APHIS' notification process, which allows for an expedited permit system for field trials of plants that meet certain criteria and with which APHIS has familiarity. Information on all the notifications and permits for release that have been submitted to APHIS is made available to the public by Information Systems for Biotechnology based at Virginia Polytechnic Institute (http://www.isb.vt.edu/). By the end of 2004, approximately 350 tree notifications covering 19 species had been acknowledged by APHIS. Overall, trees account for less than 2.5% of the approximately 14 500 Notifications acknowledged by APHIS. About 25% of the tree notifications were for interstate movement (also covered under the notification process), e.g., exchange of materials from one researcher to another, and did not result in a field trial. Forest trees species account for about two-thirds of the tree notifications, with the rest being fruit or nut trees. Probably because of its relative ease in transformation and propagation, poplar accounts for over one-third of the total notifications for tree species. About 25% of the tree notifications involve marker genes (mostly the GUS marker gene). Other traits being investigated include herbicide tolerance, insect and disease resistance, and product quality traits such as altered lignin or modified fruit ripening. To date, only one tree species, papaya, made resistant to papaya ringspot virus (PRSV), has been granted nonregulated status for planting in Hawaii. Prior to deregulation of transgenic papaya in 1996, the virus was decimating the Hawaiian papaya industry and all other efforts to control it had been ineffective. Petitions submitted to APHIS in 2004 for additional virus-resistant papayas and plums resistant to the plum pox virus are currently under review.

Conclusions

The productivity of plantation forests has benefited enormously from development and implementation of improved silvicultural and forest management practices during the past century. A second wave of improvements has been brought about by the introduction of new germplasm developed through genetics and breeding efforts for both hardwood and conifer tree species. Coupled with the genetic gains achieved through tree breeding, the emergence of new biotechnological approaches that span the field of plant morphogenesis, genetic transformation, and discovery of genes associated with complex multigenic traits have added a new dimension to forest tree improvement programs.

Significant progress has been made during the past 5 yr in the area of plant regeneration via organogenesis and SE for economically important tree species. These advances have not only helped us in developing the efficient gene transfer techniques via biolistics and *Agrobacterium*-mediated methods but also opened up the avenues for deployment of improved clonal stocks in the field. Plant regeneration and transformation challenges lie ahead in extending these methods to elite germplasm and developing cost-effective means for delivery of improved nontransgenic as well as transgenic planting stocks.

Sustainable tree plantations that can eliminate the need for harvest from natural tree stands will require the use of improved planting stock. The domestication of trees through traditional breeding has been slow, due to their perennial nature and the relatively long time to reproductive competency, which delay genetic crossing and phenotypic evaluation of seedling performance. It will be possible to accelerate the domestication process by the application of biotechnology in conjunction with traditional breeding. Improved planting stock will depend on synergy generated from specific genotype selections and propagation from elite tree-breeding programs. This selection will be made more effective through the use of molecular markers that are associated with candidate genes and desirable phenotypes. In addition, the wood derived from trees can be made more valuable through genetic modification and clonal selection. Transgenic technology may also be the most sustainable approach to protecting plantation forests from biotic and abiotic stresses.

Recent breakthroughs in genomics and gene cloning techniques have dramatically accelerated our ability to discover and introduce value-added traits for wood quality and resistance to insect pests and abiotic stresses into improved genotypes. It is predicted that the rate of publication of new discoveries in the application of molecular markers and transgene technology to tree improvement will escalate dramatically in the next 5–10 yr. It is very conceivable that the first transgenic forest tree product will be commercialized within the next 8–10 yr.

As improved trees are introduced into both small- and large-scale field tests and into commercial operations, it is important that a reliable infrastructure is in place for assessing the safety of these trees. Regulatory guidelines and requirements for testing and release of trees improved through biotechnology exist in the US and other developed nations and similar regulations are being established in many other countries around the world.

The commercializing of genetically modified trees that would further enhance the quality and productivity of our plantation forests is anticipated in the foreseeable future. Improved planting stock, obtained through the assistance of biotechnology in the development of new selected clonal varieties and in the development of transgenics, will allow the world to meet its ever-growing need for wood products grown in a sustainable fashion. Trees, as a renewable source of energy and biomaterials, have the potential to decrease our dependency on other nonrenewal sources such as oil and coal. In addition, they have the potential to sequester CO_2 and foster opportunities to recapture CO_2 from industrial sources. As tree plantations become more productive on less land, they will lessen the need for harvest from natural forest stands.

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